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**Taste compound – nanocellulose interaction assessment by
fluorescence indicator displacement assay**

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Abstract

Interactions between taste compounds and nanofibrillar cellulose were studied. For this, a new fluorescent indicator displacement method was developed. Two fluorescent indicators, namely, Calcofluor white and Congo red, were chosen because of their specific binding to cellulose and intrinsic fluorescence. Seven taste molecules with different structures and properties were successfully measured together with NFC and ranked according to their binding constants. The most pronounced interactions were found between quinine and nanofibrillar cellulose ($1.4 \times 10^4 \text{ M}^{-1}$) whereas sucrose, aspartame and glutamic acid did not bind at all. Naringin showed moderate binding while stevioside and caffeine exhibited low binding. The comparison with microcrystalline cellulose indicates that larger surface area of nanofibrillated cellulose enables stronger binding between the binder and macromolecules. The developed method can be further utilized to study interactions with different compound classes with nanocellulose materials for purposes of food, pharmaceutical and dye industries using a conventional plate reader in a high-throughput manner.

32	Keywords
33	Nanofibrillar cellulose; fluorescence indicator displacement assay; taste
34	

1. Introduction

Nanocellulose materials represent a class of cellulose materials with at least one nanoscale dimension produced either with enzymatic, chemical or physical methods from natural cellulose fibers (Klemm, Kramer, Moritz, Lindström, Ankerfors, & Gray, 2011). Nowadays they have many uses for example as bioabsorbent in wastewater treatment and in biomedical applications, drug delivery systems, tissue engineering and wound dressings (Ngwabebhoh, & Yildiz, 2019). In particular, the utilization of nanocellulose as a food additive was one of the first applications proposed (Turbak, Snyder, & Sandberg, 1983a). High surface area and aspect ratio, suitable rheological behaviour (high viscosity even at low concentrations) and the easiness of chemical modifications are advantageous for the applications in food industry, particularly in food packaging (Gómez et al., 2016; Klemm et al., 2011).

Since 1980s, many food related applications utilizing nanocellulose have been developed. In a review by Gómez et al. (2016) the applications in food science were divided in three groups: 1) as a food stabilizer, 2) as a functional food ingredient, and 3) in food packaging. As a stabilizing agent, nanocellulose materials have been used in various different food products such as in fat and oil containing products (gravies, salad dressings, and whipped toppings) (Turbak, Snyder, & Sandberg, 1982, 1983a, 1983b). Furthermore, it has been used in this purpose to prevent the spreading of cookie fillings (Kleinschmidt, 1988), to improve the shape retention of frozen desserts (Yano, Abe, Kase, Kikkawa, & Onishi, 2012) and most recently, in the shape retention of ice cream (Velásquez-Cock, et al., 2019). In functional

foods, nanocellulose materials have been used in low-calorie applications in products with high-energy content such as hamburgers (Ström, Öhgren, & Ankerfors, 2013) and to replace fats in food formulations and thus reduce their energy density (Cantiani, Knipper, & Vaslin, 2002). Furthermore, nanocellulose materials have showed promising characteristics as dietary fibers (Andrade, Mendonça, Helm, Magalhães, Muniz, & Kestur, 2015).

In food packaging applications, nanocellulose materials offer a nature-friendly option to fossil fuel based and non-biodegradable materials (Azeredo, Rosa, & Mattoso, 2017). Nanocellulose materials can act as high air and oxygen barrier, which makes them competitive to other packaging materials (Aulin, Gällstedt, & Lindström, 2010; Gómez et al., 2016). They can also serve as carriers for active substances in food packaging applications (Huq et al., 2012). For example, Lavoine, Desloges and Bras (2014) used a paper coated with microfibrillated cellulose for the controlled release of caffeine, whereas Jipa, Stoica-Guzun and Stroescu (2012) studied controlled release of sorbic acid from bacterial cellulose films.

In this study, the aim was to evaluate the interactions between nanocellulose materials and taste compounds. Despite the many food related applications of nanocellulose materials, to our knowledge there are no systematic studies about the possible effects of nanocellulose to the taste of food. Troszyńska et al. (2010) studied the effect of food gums (i.e. guar, xanthan, arabic) and carboxymethylcellulose (CMC) on the astringency induced by phenolic compounds. According to their study, CMC was the best at masking astringency. Furthermore, the interactions between nanocellulose materials and drug molecules have been studied. Particularly, Kolakovic et al. (2013)

used isothermal titration calorimetry (ITC) and an incubation method (incubation of drug molecules with NFC, centrifugation and quantification of an unbound drug from supernatant) to study the binding of drug compounds to nanofibrillated cellulose (NFC). In similar manner, Jackson, Letchford, Wasserman, Ye, Hamad, & Burt (2011) studied the binding of drug molecules to nanocrystalline cellulose (NCC) by measuring the amount of unbound molecules by using a spectrophotometry method.

The methodologies presented above are accurate, but time-consuming and molecule dependent. For each compound, a new or at least refined methodology is needed. In contrast, a more generic method based on fluorescent indicator (FI) displacement for nanocellulose-taste compound interaction assessment is developed in this study. With this method, it is possible to screen a wide spectrum of molecules with different characteristics with one method using a plate reader with e.g. a 96-well plates. Thus, the developed method is both affordable and efficient. The method is based on the competitive binding of a well-known FI molecule and a second molecule, whose binding to a macromolecule, in this case to NFC, is investigated. If the interaction between molecule of interest and NFC occurs, a decrease of FI fluorescence intensity can be detected as it is displaced from the fiber surface. Similar methods have been used before for example in the assessment interactions of different analytes to DNA, RNA and proteins (Asare-Okai, & Chow, 2011; Ham, Winston, & Boger, 2003; Mock. Langford, Dubois, Criscimagna, & Horowitz, 1985; Zhang, Umemoto, & Nakatani, 2010). These methodologies have been reviewed by Nguyen & Anslyn (2006) and Tse, & Boger, 2004. Nevertheless, to our knowledge these methods have not

been used before to assess macromolecule interactions with taste compounds. Two FIs were chosen based on their specific binding to cellulose (Wood, 1980) and different photophysical properties to avoid a possible situation where the molecule of interest absorbs light at the same wavelength that is used to excite the FI. Calcofluor white has its absorption maximum at around 350 nm while the absorption maximum of Congo red is at around 500 nm (Wood, 1980). With these indicators, a wide variety of taste compounds with different taste characteristics could be studied. Seven taste compounds, caffeine, aspartame, quinine, stevioside, sucrose, naringin and glutamic acid, with different taste characteristics (sweet, bitter, umami) were chosen for this study. Salts and strongly acidic compounds were excluded from the study as salts and extreme pH causes swelling of cellulose materials (Grignon, & Scallan, 1980).

2. Materials and Methods

2.1. Materials

Cellulose nanofibrils (dimeric unit presented in the Figure 1a) were obtained from UPM Corporation (Finland) as a 1.5 wt % hydrogel. Microfibrillated cellulose (MCC, Avicel®, Sigma-Aldrich) was used as a 1.5 wt % suspension prepared with water purified with a Milli-Q system (Millipore, Burlington, Massachusetts, USA). The FIs used were Fluorescence brightener 28 (Calcofluor white M2R) (Figure 1b) from Sigma-Aldrich (St. Louis, MO, USA) and Congo Red (> 98 %) from Tokyo Chemical Industry CO., LTD. (Tokyo, Japan) (Figure 1c).

The studied taste compounds (Figure 1 d-j) were caffeine (99 %), naringin and aspartame (98 %) from ThermoFisher GmbH (Kandel, Germany) and glutamic acid (99 %), stevioside, sucrose (> 99 %) and quinine (99 %) from Acros Organics (Geel, Belgium). The compounds were chosen based on their known taste properties to include compounds, which either create a pleasant taste (sweet and umami) or have related unpleasant characteristics (bitter).

2.2.Methods

2.2.1. UV-Vis characterizations

Water solutions of the fluorescent indicators i.e. calcofluor white (CFW) and Congo red (CR) were measured with UV-Vis-NIR spectrophotometer (UV-3600, Shimadzu) in 1 cm² standard quartz cuvettes. Absorption spectra were measured from 250 to 600 nm varying the concentration from 0 to 26 µM for both calcofluor white and Congo Red. MQ-water was used to adjust the samples concentrations. Absorption maxima were detected at 349 nm and 499 nm for CFW and CR, respectively. Molar extinction coefficients were calculated based on the absorption measurements.

2.2.2. Titration of the fluorescent indicator with nanofibrillar cellulose

Fluorescent indicators CFW and CR in concentrations of 6 µM and 2.5 µM, respectively, were titrated with a NFC hydrogel to a final NFC concentration of 0.04 M. The concentration of FIs were chosen to avoid inner filter effects on the fluorescence of FIs. As the molecular weight of NFC macromolecules varies, the concentration of NFC is represented in moles of monomeric cellulose units per liter using 162.14 g/mol as the molar mass of the monomer. This practice is used commonly with biopolymers such as DNA and RNA

where the concentration is expressed as the concentration of nucleobases or pairs of nucleobases. The changes in the fluorescence intensity of CFW upon titration with NFC were measured in triplicates by spectrofluorometer Fluorolog-3® (Jobin Yvon) or plate reader Fluoroskan Ascent FL (Thermo Labsystems). The changes in the fluorescence intensity of CR upon titration with NFC were measured in triplicates by using a plate reader. The excitation/emission filter pairs for measurement with spectrofluorometric plate reader were chosen to be 355/460 nm for CFW and 485/590 nm with CR based on their absorption/emission spectra. The titration of MCC with CR was conducted in a similar manner as with NFC with concentration range from 0.002 to 0.088 M. MCC concentration was estimated in the same way as for NFC.

The binding constants (K_{bind}) for FIs with NFC were calculated using Benesi-Hildebrand method (Benesi & Hildebrand, 1949) as follows:

$$\frac{I_{max}-I_{free}}{I_n-I_{free}} = 1 + \frac{1}{K_{bind}[NFC]} \quad (1)$$

where [NFC] is the added NFC concentrations, I_{max} is the maximum fluorescence intensity of FI in the presence of NFC when the saturation is reached, I_{free} is the fluorescence intensity of FI in the absence of NFC, I_n is the fluorescence intensity of FI in the presence of NFC at an intermediate concentration and K_{bind} is the binding constant for the FI. By plotting $\frac{I_{max}-I_{free}}{I_n-I_{free}}$ versus $1/[NFC]$ the values of K_{bind} were obtained from the slope of the linear fit.

2.2.3. Titration of pre-formed fluorescent indicator-nanofibrillar cellulose complex with taste compounds

All the samples contained either 0.04 M of NFC with 6 μ M CFW or 0.025 M of NFC with 2.5 μ M CR and varying concentrations of the taste compounds (Table 1). The concentration ranges for the taste compounds were chosen based on their solubility in water. All solutions were mixed carefully to avoid bubbles. FI for each compound was chosen based on their photophysical characteristics, i.e. whether they would absorb light at the excitation wavelength of the FI or not. In order to estimate possible errors by using different FIs, cross-validation of caffeine-NFC interaction was studied by using both CFW and CR. 150 μ L of each sample solution was pipetted on a well plate and measured with plate reader as above (2.2.2.). Each taste compound was studied as triplicates.

The binding constants were determined with Benesi-Hildebrand method as before. As the substitution of FI causes decreasing fluorescence intensity, equation 1 was modified as follows:

$$\frac{I_0 - I_{free}}{I_0 - I_n} = 1 + \frac{1}{K_{bind}[TC]} \quad (2)$$

[TC] is the added taste compound concentrations, K_{bind} is the binding constant of the taste compound, I_0 is the fluorescence intensity of FI-NFC mixture in the absence of the taste compounds, I_{free} is the fluorescence intensity of FI in the absence of NFC, I_n is the fluorescence intensity of FI-NFC mixture in the presence of the taste compounds at an intermediate concentration.

2.2.4. Cross-validation with ITC

Isothermal titration calorimetry was performed using a Microcal VP-ITC (GE Healthcare, Life Sciences, MicroCal, Northampton, MA). A sample cell was filled with quinine (0.39 mM). Experiments were carried out at 25 °C by injecting 20 µL of 15 mM NFC sample solution 15 times. As control measurements, MQ was titrated with 15 mM NFC and 0.39 mM quinine with MQ. The differential enthalpy curves of heat of titration of MQ with NFC and the averaged enthalpy of titration of quinine with MQ were then subtracted from the curves of binding of quinine to NFC. Data analysis was performed with Microcal Origin software and one binding site model was used for fitting.

3. Results and Discussion

3.1. Binding constants of fluorescence indicators

Based on the results of spectrophotometry, the molar extinction coefficients in water for CFW and CR were calculated to be $\epsilon_{\text{CFW}}(349 \text{ nm}) = 53.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{\text{CR}}(499 \text{ nm}) = 38.9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. Thus, indicator concentrations of 6 µM and 2.5 µM for CFW and CR were used for the titration experiments with NFC to neglect the inner filter effect on the fluorescence. During the titrations of CFW and CR with NFC an increase in the fluorescence intensity of both dyes was observed, indicating that both FIs bind to NFC. The fluorescence intensities of CFW and CR against the added NFC concentration are presented in Figure 2. The data is also presented according to Eq. (1) to calculate the binding constants (Figure 2, insets).

The obtained binding constants were $27 \pm 7 \text{ M}^{-1}$ for CFW and $58 \pm 12 \text{ M}^{-1}$ for CR. Based on the saturation curves (Figure 2), NFC concentrations of 0.04 M with CFW and 0.025 M with CR were chosen for taste compound titrations as the saturation and maximum intensity were reached at these concentrations. When MCC was titrated with CR, saturation was not reached within the studied concentration range and the binding constant was estimated to be approximately 4 M^{-1} . This is more than 10 times lower compared to NFC-CR interaction and is probably due to the considerably lower specific surface area of MCC (ca. $1.3 \text{ m}^2/\text{g}$ for Avicel PH 102 (Ardizzzone, et al., 1999) compared to NFC ($50 - 70 \text{ m}^2/\text{g}$ (Missoum, Belgacem, & Bras, 2013)). As the binding is surface area dependent, it is logical that the binding constants are considerably lower in the case of MCC. This further means that the possible effect of binding of the taste compounds on the taste of foods can be perceived with NFC even if this is not the case with MCC-containing formulations or products.

3.2. Binding constants of taste compounds

The interaction between the pre-formed NFC-FI complexes and taste compounds resulted in a clear decrease in the fluorescence of the FIs because of FI displacement from the NFC matrix. As the FI-NFC complexes have stronger fluorescence than the free FIs, the overall fluorescence intensity in the system will decrease if the dyes are released from the NFC surface. This happens when a taste compound binds to a cellulose surface that is initially fully covered by the FI. It is good to notice that the measured signal comes from the FI in all the measurements, and not from the taste compounds. FI displacement curves are presented in Figure 3 for those taste compounds that

showed clear complex formation with NFC. These curves can be used to evaluate the binding constants according to eq. (2).

Clear trends in the fluorescence intensity can be seen in Figure 3 for caffeine, stevioside, naringin and quinine even though the experimental fluctuations are considerable especially for stevioside and naringin. The binding isotherms can be used for ranking the taste compounds in the order of binding strength and for the evaluation of the binding constants. As an example, a 20 % FI (CFW) displacement was achieved at ca. 5 mM concentration of caffeine, whilst for quinine the same percent was achieved at ca. 0.025 mM concentration. Roughly 1.8 mM and 0.2 mM concentrations for stevioside and naringin respectively were needed to reach the same displacement. Sucrose, aspartame and glutamic acid had negligible binding according to our measurements, as no clear fluorescence decrease was seen with these molecules. Finally, the binding constants of all the tested compounds calculated with Eq. (2) are presented in Table 1 as mean values of triplicates.

In order to verify the comparability of the results obtained by different FIs, the binding values for caffeine was estimated with both indicators. The slopes for caffeine-NFC interactions plotted according to Eq. (2) are close to each other. The calculated binding constant of caffeine to NFC obtained with CR was 86 M^{-1} , which is very close to the measured binding constant with CFW (70 M^{-1} , Table 1). Thus, the method can be used with either of the selected fluorescence indicators, and the indicator can be chosen based on whether the molecules have spectral overlap with the FI or not. As all seven taste compounds with different structures and properties were measured successfully using a plate reader and 96-well plates in a high-throughput

manner, it can be concluded that a similar methodology can be also utilized in future for studying larger sets of compounds for applications in e.g. food industry and pharmaceutical fields.

Isothermal titration calorimetry (ITC) was used for cross-validation of the method. As only relatively high enthalpy changes can be measured with this method, quinine with the highest binding constant to NFC was chosen for these studies. Enthalpy curve of quinine binding to NFC resulting from ITC is presented on Figure 4. The estimated binding constant for quinine measured with ITC and calculated with one binding sites model was $19\,000 \pm 5790\text{ M}^{-1}$. Based on these results it can be concluded that the binding constants achieved with fluorescence indicator displacement method are reasonably accurate and in line with results obtained with a more established ITC methods. Furthermore, with this method weaker interactions can be measured than with traditional methods like ITC.

Based on estimated binding constants, taste compounds can be divided into four groups: non-binding molecules, molecules with weak interactions, molecules with moderate interactions and molecules with distinct interactions. Of the studied compounds, sucrose, aspartame and glutamic acid belong to the group of non-binding molecules, caffeine and stevioside have weak interactions, whereas naringin has moderate interactions. Quinine has clearly more pronounced interactions than the other studied molecules, with ca. 200 times higher binding constant than caffeine for example, making it the strongest binding molecule in our test set. The measured binding constant ($14\,000\text{ M}^{-1}$) is of the same order of magnitude as was measured for

hydrophobin proteins binding to NFC (Kolakovic, et al., 2013), indicating strong binding between quinine and NFC.

From the Table 1 it can be seen, that the interactions seem to partly correlate with the aqueous solubility and octanol/water partition of the compounds. The highest binding constant was achieved with quinine, which is also the least water soluble of the studied molecules. This indicates that despite NFC hydrophilic nature, in aqueous solutions the nanofilbrillar cellulose is acting as a slightly hydrophobic target as water molecules already occupy most of its surface. Also, all the non-binding molecules have relatively high solubilities and low logP values. However, caffeine with lower logP and higher solubility has higher binding constant compared to aspartame. This might be explained by the negative charge of aspartame as well as glutamic acid in aqueous solutions (near neutral or slightly acidic conditions) lowering the probability of binding to nanofibrillar cellulose, which contains some amount of negatively charged hemicellulose on its surface (Kolakovic et al., 2013). Indeed, slightly negative zeta-potential values for NFC at pH 5 have been previously reported (Fall, Lindström, Sundman, Ödberf & Wågberg, 2011). Furthermore, Kolakovic et al. (2013) stressed the stronger interaction of NFC with positively charged drugs in comparison to neutral or anionic drug molecules, as the electrostatic interactions have a significant impact on complex formation. On the other hand, quinine with the highest binding constant has a positive charge in this pH favoring the binding. Also, the amine groups might increase the binding probability in the case of quinine and caffeine. Furthermore, it is probable that also other effects, such as hydrogen

bonding ability of the compounds, planarity and steric hindrances effect the binding.

Based on our results, the bitter tasting molecules are top-ranked in terms of their NFC binding constants. This finding indicates that NFC might be used as bitterness suppressing material in the future. Noteworthy, it has been already shown that CMC is able to mask the astringent taste of phenolic compounds (Troszyńska et al., 2010). NFC can be expected to have similar or even more pronounced effect on these compounds due to its small particle size and large surface area. Thus, this study reveals a new promising characteristic of NFC in food applications as a taste modifier besides the known uses of nanofibrillar cellulose as an emulsion stabilizer and a functional food ingredient. Despite the foreseen applications of nanocellulose and the commercial use of bacterial cellulose as food ingredient in Philippines (nata de coco), nanofibrillar cellulose has not yet been accepted as a food additive in EU or USA. This study indicates a further possibility for the utilization of this abundant biopolymer in future applications. However as stated in the literature (Gomez et al., 2016), there is still a need for rigorous safety evaluations of nanocellulose materials before its full potential can be realized.

4. Conclusions

A high-throughput screening method utilizing a plate-reader was developed for the estimation of binding constants of taste molecules with NFC. In this study, binding constants between 70 M^{-1} and $14\,000 \text{ M}^{-1}$ were measured with good accuracy. The method seems promising for looking at the binding of

taste compounds but also as a generic interaction assay. The studied taste compounds were divided into four groups based on their interaction strengths. Non-binding molecules were sucrose, aspartame and glutamic acid. Caffeine and stevioside were weak binders whereas naringin was a moderate NFC ligand. The bitter tasting quinine was the strongest binder in the set of the molecules studied. The magnitudes of the binding strengths seem to be at least partly correlated to the hydrophobicity of compounds. As the bitter tasting compounds are among the best NFC binders in the set, the finding can be usefull for the development of bitter suppressing or masking applications both in food and pharmaceutical industries. This should be further studied with sensory analysis to evaluate the real effects of these interactions on perceived taste.

Abbreviations

NFC nanofibrillar cellulose

FI fluorescence indicator

CR congo red

CFW calcofluor white

MQ Milli-Q water

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Tables

Table 1. Binding constants of taste compounds to NFC as mean values with standard deviations (n = 3). Compounds are grouped based on their taste characteristics. For each compounds, molar mass, solubility to water and logP value is provided. negl. = negligible. ¹ Windholz, 1983; ² Furia; 1980; ³ Mazzobre, Roman, Mourelle, & Corti, 2005; ⁴ Dreisewerd, Merz, & Schembecker, 2015, ⁵ Cargill, Inc, 2010; ⁶ Valko, Bevan, Reynolds, & Abraham, 2000; ⁷ Yuan, Liu, Xiao, Leng, Liao, Ma, Liu, 2019); ⁸ Rankovic, 2017; ⁹ Hansch, Leo, & Hoekman, 1995;

Taste	Compound	FI	MW [g/mol]	Solubility to water [mg/ml]	Log P	K _{bind} , M ⁻¹
Sweet	Sucrose	CFW	342.30	2 000 ¹	-3.3 ³	negl.
	Stevioside	CFW	804.88	1.25 ¹	1.19 ⁴	146 ± 34
	Aspartame	CFW	294.31	10.20 ²	0.07 ⁵	negl.
Bitter	Caffeine	CFW (CR)	194.19	21.74 ¹	-0.07 ⁶	70 ± 25 (86)
	Naringin	CFW	580.53	1.00 ¹	-0.5 ⁷	1251 ± 385
	Quinine	CR	324.42	0.53 ¹	2.51 ⁸	14300 ± 1500
Umami	Glutamic acid	CFW	147.13	8640 ¹	-3.69 ⁹	negl.

Figure captions

Figure 1. Chemical structures of Cellulose (a), the FIs Calcofluor white (b) and Congo red (c), and the studied taste compounds: aspartame (d), caffeine (e) glutamic acid (f), naringin (g), quinine (h), sucrose (i) and stevioside (j).

Figure 2. Examples of the saturation curves for titration of the FIs with NFC, *i.e.* the fluorescence intensity of CFW (6 μ M) (a) and CR (2.5 μ M) (b) as a function of NFC concentration. The reciprocal plots (Eq. 1, $\theta = \frac{I_{max}-I_{free}}{I_n-I_{free}}$) are presented in insets.

Figure 3. Examples of the saturation curves for the binding of each taste compound to NFC *i.e.* the fraction of bound FI as a function of taste compound concentration with estimated trend-lines to help reading. For caffeine (a), both CFW and CR were used as FI. For stevioside (b) and naringin (c) CFW was used as FI and for quinine (d) CR was chosen as FI. Reciprocal plots (Eq. 2, $\alpha = \frac{I_0-I_{free}}{I_0-I_n}$) are presented as insets.

Figure 4. Enthalpy curve of titration of quinine with NFC